Conjugated linoleic acid and fatty acid binding protein as antioxidants

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SUMMARY

Studies were carried out to determine the effect of conjugated linoleic acid (CLA) and rat liver cytosolic protein enriched in fatty acid binding protein (FABP) on the non-enzymatic lipid peroxidation of rat liver microsomes. The inhibition of lipid peroxidation was more evident when the FABP containing fraction obtained from CLA-group was used with either kind of microsomes (CLA and control). The chemiluminescence and polyunsaturated fatty acid composition of rat liver microsomes changed after CLA treatment. When native and peroxidized microsomes obtained from control group were compared, the most affected polyunsaturated fatty acids were: C18:2, C18:3 and C20:4, while in CLA-group C20:4 was mainly peroxidized The simultaneous analysis of chemiluminescence and fatty acid composition demonstrated that CLA and FABP play a role protecting rat liver microsomes against the harmful effect of lipid peroxidation.

Key words: (Conjugated Linoleic Acid), (Fatty Acid Binding Protein), (lipid peroxidation), (liver), (microsomes)

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Ácido linoleico conjugado y proteína transportadora de ácidos grasos como antioxidantes

RESUMEN

El objetivo del trabajo fue determinar el efecto del ácido linoleico conjugado (ALC) y de la proteína citosólica de hígado de rata enriquecida en Proteína Transportadora de Ácidos Grasos (PTAG), sobre la peroxidación no enzimática de lípidos de microsomas hepáticos de rata. Luego de la incubación de éstos en un sistema ascorbato-Fe^{++} se observó que el total de cpm/mg de proteina originada por quimioluminiscencia fue menor en los microsomas obtenidos de las ratas del grupo ALC respecto a los del grupo control. Cuando la fracción PTAG obtenida del grupo ALC fue agregada a la peroxidación de microsomas de ambos grupos de animales ALC y control, la inhibición de la lipoperoxidación fue más evidente. Además se encontró que ambas fracciones PTAG, tanto la obtenida de animales del grupo ALC como la obtenida del grupo control, tuvieron mayor efecto como antioxidantes cuando se usaron microsomas ALC respecto a microsomas control. La composición de ácidos grasos de los microsomas cambió luego del tratamiento con ALC. Comparando microsomas nativos y peroxidados obtenidos de grupo control, los ácidos grasos polinosaturados afectados fueron: C18:2 (n-6), C18:3 (n-3) y C20:4 (n-6), y en el grupo ALC fue C20:4 (n-6). El análisis de quimioluminiscencia y composición de ácidos grasos demostró que tanto el ALC como la PTAG juegan un rol en la protección de los microsomas de hígado de rata contra los efectos nocivos de la lipoperoxidación.

Palabras clave: (Ácido linoleico conjugado), (proteína transportadora de ácidos grasos), (peroxidación lipídica), (hígado), (microsomas).

INTRODUCTION

The oxidative degradation of polyunsaturated fatty acids in bio-membranes can be initiated by free radicals and can proceed in a chain reaction causing considerable membrane damage or producing lipid peroxides[31]. Conjugated linoleic acid (CLA) is a collective term referring generally to a mixture of positional and geometric conjugated dienoic isomers of linoleic acid. Numerous CLA isomers are found in milk-fat, cheese, and beef [9,29]. The cis-9, trans-11, isomer of CLA (the principal dietary form) is produced in the rumen of cattle and other ruminant animals during the microbial biohydrogenation of linoleic and linolenic acids [13,20,23,36,39,42]. It is evident that CLA significantly reduced tumor incidence as well as tumor cell proliferation[14-16,19]. The mechanisms through which CLA inhibits tumorigenesis are moot.[32]. In different studies it has been demonstrated that CLA is a potent antioxidant and that c-9, t-11 isomer is selectively incorporated into cellular phospholipid, which may, at least, in part, explain the anticarcinogenic activity of CLA as antioxidant mechanism[33]. One might speculate that the inhibition of carcinogenesis by CLA could result from the combined effects of a number of CLA activities, possibly including

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direct effects of one or more CLA isomers/ metabolites on cell differentiation and effects of one or more CLA isomers on prostaglandin metabolism which may also influence cancer development at some sites[5,27,34]. Animal fat, which has been maligned for so long, may actually constitute a potent therapeutic component to our diet[11, 24]. In rat liver, 60 % of cytosolic long chain fatty acids are associated with a low molecular weight protein 15 KDa named Fatty Acid Binding Protein (FABP) [7]. In vitro studies of our laboratory have shown that cytosolic fraction enriched with FABP contains antioxidant properties against lipid peroxidation [8]. For this reason, it was of interest to analyze the effect of this protein on the lipid peroxidation process of liver microsomes isolated from rat treated with CLA. The present study aims to compare the polyunsaturated fatty acid composition and non enzymatic lipid peroxidation of rat liver microsomes obtained from animals supplemented with conjugated linoleic acid with control animals, and to analyze the effect of Fatty Acid Binding Protein on the lipid peroxidation process.

MATERIAL AND METHODS

Female Wistar AH/HOK rats were obtained from Laboratory Animal Facility, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. Octadecadienoic acid conjugated (CLA) and butylated hydroxytoluene (BHT) were from the Sigma Chemical Co. (St. Louis, MO). BSA (fractionV) was obtained from Wako Pure Chemicals Industries Ltd; Japan. Standards of fatty acids methyl esters were generously supplied by NU Check Prep, Inc, Elysian, MN, USA. L [+] ascorbic acid and boron trifluoride-methanol complex were from Merck. All other reagents and chemicals were of analytical grade from Sigma.

1. Animal treatment

Seven- week- old female Wistar AH/HOK rats, weighing 120-137 g were used. Two groups of five rats were considered, Group A received conjugated linoleic acid (CLA), Group B was used as control. All rats were fed commercial rat chow and water ad-libitum. Group A received a daily oral dose of CLA (30 mg) for 10 days. On day 11, rats were sacrificed by cervical dislocation and the liver was rapidly removed.

2. Preparation of microsomes and cytosolic fraction enriched in fatty acid binding protein

Liver was cut into small pieces and washed extensively with 0.15 M NaCl. A homogenate 30% (w/v) was prepared in a solution (0.25 M sucrose, 10 mM Tris-HCl pH 7.4) using the Potter-Elvejhem homogenizer. The homogenate was spun at 10,000 g for 10 min. The supernatant (3 ml) obtained was applied to a Sepharose 4B column (1.6 x 12 cm) equilibrated and eluted with 10 mM Tris-HCl (pH 7.4), 0.01 % NaN_3. The microsomal fraction appearing in the void volume (8-12 ml) was brought to 0.25 M sucrose by addition of solid sucrose. With respect to concentrations and activities of certain microsomal enzymes, the quality of this microsomal preparation has a similar composition to that obtained by ultracentrifugation[41].

The soluble fraction (14-22 ml) was concentrated and filtrated on Sephadex G-75 (3x39 cm) eluted with buffer Tris-HCl 0.01 M, pH 7.4, 0.01% NaN3, flow rate: 90 ml/h, to obtain two large fractions F1 and F2. F1 corresponded to proteins with molecular weight greater than 68 KDa and F2 represented proteins in the range 12-16 KDa that are enriched with FABP, this fraction was concentrated by ultrafiltration in an Amicon

cell containing a PM10 membrane. All operations were performed at 4 °C and under dim light.

3. Lipid peroxidation of rat liver microsomes

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to microsomes [44]. Microsomes (1 mg protein) were incubated at 37 °C with 0.01M phosphate buffer pH 7.4, 0.4 mM ascorbate, final volume 2 ml. Phosphate buffer was contaminated with sufficient iron to provide the necessary ferrous or ferric iron for lipid peroxidation [40]. In each assay, 0.25, 0.50 and 1 mg of protein of FABP obtained from two groups (ALC and control) were used. Organelle preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 120 min period, chemiluminescence was recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per min in a liquid scintillation analyzer Packard 1900 TR equipment with a program for chemiluminescence.

4. Fatty acid analysis

Microsomal lipids were extracted with chloroform/methanol (2:1 v/v) [12] from native or peroxidized membranes. Fatty acids were transmethylated with F_3B in methanol at 60 °C for 3 h. Fatty acids methyl esters were analyzed with a GC-14 A gas chromatograph (Shimadzu Kyoto, Japan) equipped with a packed column (1.80 m x 4 mm i.d.) GP 10 % DEGS-PS on 80/100 Supelcoport. Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250 °C, the column temperature was held at 200 °C. Fatty acid methyl ester peaks were identified by comparison of the retention times with those of standard.

5. Other methods

Proteins were determined by Lowry's method [28].

6. Statistical analysis

Results are expressed as mean ± S.D. of five independent determinations. Data were evaluated statistically by one-way analysis of variance (ANOVA) and Tukey test. The statistical criterion for significance was selected at different P values and indicated in each case.

RESULTS

The polyunsaturated fatty acid composition of total lipids isolated from rat liver microsomes obtained from CLA and control groups is shown in Table 1. The effect of conjugated linoleic acid on the total fatty acid composition of native microsomes showed a significant decrease in linoleic acid C18:2 (n-6) when compared with the control group. When peroxidized microsomes were compared with natives, the arachidonic acid C20:4 (n-6) decreased in the two groups, the linoleic acid C18:2 (n-6) and the linolenic acid C18:3 (n-3) decreased only in the control group. Statistically significant differences in the content of C18:2 (n-6), C18:3 (n-3) and C20:4 (n-6) with respect to the control group were observed in CLA-group when peroxidized microsomes were compared.

The incubation of rat liver microsomes in the presence of ascorbate -Fe^{++} resulted in the peroxidation of membranes as evidenced by the emission of light (chemiluminescence). The lipid peroxidation process measured as light emission decreased when microsomes isolated from CLA-group were compared with

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Table 1. Polyunsaturated fatty acid composition (area %) of rat liver microsomes native and peroxidized from CLA and control groups

Fatty acid	CLA group		Control group	
	Native	Peroxidized	Native	Peroxidized
18:2 n-6	7.232 ± 0.751[h]	5.406 ± 2.316[e]	9.374 ± 1.064[b,h]	1.516 ± 0.128[b,e]
18:3 n-3	0.715 ± 0.316	0.554 ± 0.216[f]	1.275 ± 0.516[c]	tr[c,f]
20:4 n-6	19.915 ± 4.480[a]	10.012 ± 2.543[a,g]	21.115 ± 4.031[d]	3.796 ±1.523[d,g]
22:6 n-3	tr	tr	tr	tr

Data are given as the mean ± S.D. of five experiments. Native: without incubation in ascorbate Fe^{++} system; peroxidized: with incubation in the presence of 0.4 mM ascorbate. Statistically significant differences between native vs. peroxidized microsomes of the two groups are indicated by $p^a < 0.03$, p^b and $p^c < 0.0001$, $p^d < 0.002$. Statistically significant differences between peroxidized microsomes are indicated by $p^e < 0.04$, $p^f < 0.05$ and $p^g < 0.01$. Statistically significant differences between native microsomes of the two groups are indicated by $p^h < 0.05$. tr: trace.

Table 2. Lipid Peroxidation of rat liver microsomes-control. Effect of FABP containing fraction obtained from control and CLA treated groups.

Microsomes	FABP fraction (mg)	Light emission (cpmx10^{-3})	Inhibition %
Native	00	2300± 205	00
+FABP control	0.25	1955 ± 172	15 ± 2
	0.50	1655 ± 102	43 ± 5 [b]
	1.00	1311 ± 133	28 ± 4 [a]
+ FABP-CLA	0.25	1840 ± 230	40 ± 5 [a]
	0.50	1380 ± 178	20 ± 3
	1.00	966 ± 127	58 ± 4 [b]

Data are given as the mean ± S.D. of five experiments. Statistically significant differences between control and supplemented groups are indicated by $p^a < 0.03$ and $p^b < 0.02$.

microsomes obtained from the control group. Moreover, light emission was inhibited by addition of the FABP containing fraction; significant differences were found when the activity of FABP from CLA and the control group was compared. The inhibition of lipid peroxidation microsomes (obtained from CLA and control groups) as a function of FABP containing fraction is illustrated in Tables 2 and 3 respectively. In both cases inhibition of light emission by the FABP containing fraction was protein concentration dependent. As shown in Tables 2 and 3, the inhibition of lipid peroxidation was more evident when the FABP containing fraction obtained from CLA treated group was used in either kind of microsomes (CLA and control).

Fig 1 shows the total light emission in the organelles obtained after lipid peroxidation of microsomes from CLA and control groups. Both FABPs (CLA and control) were more effective as antioxidant using CLA rather than control microsomes. These results are illustrated when comparing Fig. 1A with Fig.1B statistically.

Table 3. Lipid Peroxidation of rat liver microsomes-CLA. Effect of FABP containing fraction obtained from control and CLA treated groups.

Microsomes	**FABP fraction** (mg)	**Light emission** (cpmx10^{-3})	**Inhibition** %
Natives	00	1349 ± 196	00
+FABP control	0.25	1052 ± 210	22 ± 3[a]
	0.50	715 ± 92	47 ± 5[b]
	1.00	499 ± 107	63 ± 7
+ FABP-CLA	0.25	768 ± 197	43 ± 4 [a]
	0.50	418 ± 89	69 ± 7 [b]
	1.00	378 ± 72	72 ± 6

Data are given as the mean ± S.D. of five experiments. Statistically significant differences between control and supplemented groups are indicated by $p^a < 0.002$ and $p^b < 0.01$.

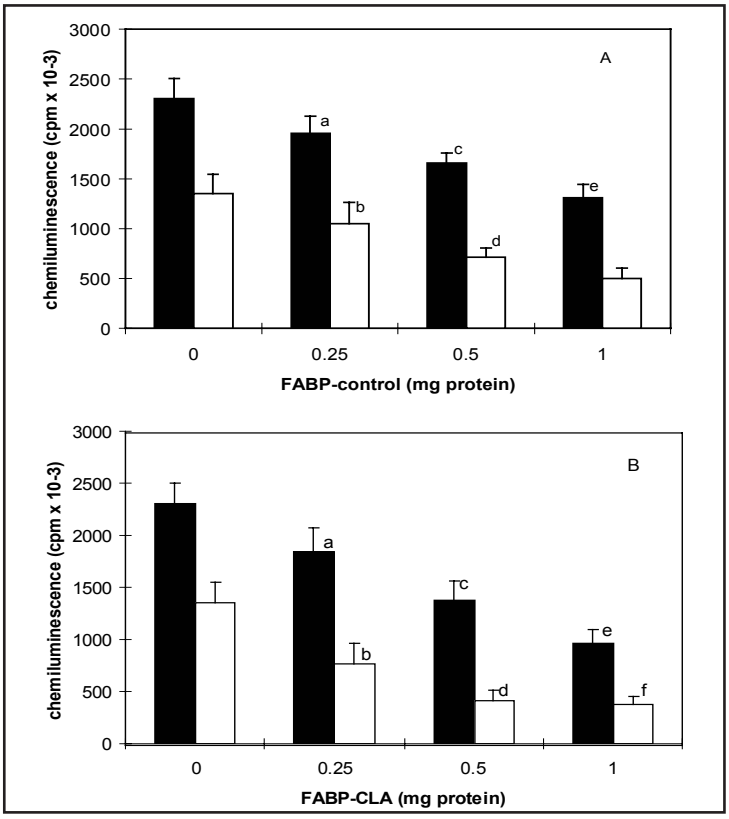

Fig.1. Effect of fatty acid binding protein-containing fraction obtained from control (A) and CLA (B) groups, on ascorbate-Fe^{++} lipid peroxidation induced in vitro. Microsomal membranes from control (■) and CLA (□) supplemented animals. Data are given as the mean ± S.D. of five experiments. Statistically significant differences between control and supplemented FABP in lipid peroxidation of microsomes are indicated by $p^a < 0.006$, p^b, p^c and $p^e < 0.001$, $p^d < 0.004$ and $p^f < 0.002$.

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DISCUSSION

Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation[18, 22,37,38,43], it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. Evidence leading to the recognition of the anticarcinogenic activity of the conjugated dienoic derivatives of linoleic acid (CLA) has been reviewed. New data indicated that CLA has a potent antioxidant activity [19,21]. Because the c-9, t-11 CLA isomer is esterified in phospholipid, it may represent a heretofore-unrecognized *in situ* defense mechanism against membrane attack by oxygen radicals [4,33]. It appears to act as a chain-breaking antioxidant by trapping chain propagating free radicals [6,17]. The carcinogenesis inhibition could result from the combined effects of CLA activities [1]. Lipid peroxidation studies *in vitro* are useful for the elucidation of possible mechanism of peroxide formation *in vivo* [3,25] since the high concentration of polyunsaturated fatty acids in membranes causes susceptibility to lipid peroxidative degradation [35]. The present study showed that rat liver microsomes obtained from CLA group are protected against lipid peroxidation when compared with similar membranes obtained from control rats, as revealed by the results obtained by chemiluminescence and polyunsaturated fatty acid composition. Moreover, significant differences of fatty acid composition between CLA with respect to the control group were found. Our results showed that CLA decreases polyunsaturated fatty acids, for this reason another author evoke that CLA does not appear to act as an antioxidant, its ability to decrease polyenoic fatty acid concentration could decrease the formation of highly cytotoxic lipid peroxidation products [2]. In contrast, another investigator suggested an antioxidant mechanism of CLA [24]. Nevertheless, the same authors postulate that CLA may induce lipid peroxidation in vivo [26]. It has been recently proposed that CLA increases the concentration of FABP by activation of specific receptors. Conjugated linoleic acid is a ligand and activator of Peroxisome-Proliferator Activated Receptor (alpha) and its effects on lipid metabolism may be attributed to transcriptional events associated with this nuclear receptor. Levels of liver fatty acid-binding protein were induced by CLA in hepatoma cells [10,30]. We agree that perhaps this mechanism is involved in improving the antioxidant activity of FABP, because the inhibition of light emission by FABP was protein concentration dependent. In conclusion, our results are consistent with the hypothesis that formation and action of CLA represents a previously unrecognized *in situ* defense mechanism against membrane attack by oxygen radicals. Further studies are needed to more adequately evaluate these observations.

CONCLUSIONS

The oxidative degradation of polyunsaturated fatty acids in bio-membranes can be initiated by free radicals and can proceed in a chain reaction causing considerable membrane damage or producing lipid peroxides. This article is a contribution to knowledge in the field. Studies were carried out to determine the effect of CLA and rat liver cytosolic protein enriched in FABP on the non-enzymatic lipid peroxidation of rat liver microsomes.

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